REPRODUCTION OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS AT LOW IONIC STRENGTH

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REPRODUCTION OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS AT LOW IONIC STRENGTH

T. M. Sokolova, I. B. Tazulakhova, S. S. Grigoryan, F. I. Ershov

The influence of low ionic strength on reproduction of Venezuelan equine encephalomyelitis virus was studied. Low ionic strength was found to inhibit reversibly the process of virus release from cells at all stages of virus reproduction. Under hypotonic conditions synthesis of viral RNA and proteins occurred at the normal rate. Virus structures formed in the cells under normal and hypotonic conditions were analysed. It was shown that in the logarythmic period at low ionic strength virus ribonucleoproteins were formed and accumulated in the cells which were probably bound with cell membranes slower than under normal conditions. The evidence is presented that at the end of the virus cycle in hypotonic medium intermediate virus forms accumulate on membranes and apparently virions with proper sedimentation constant are formed which under these conditions may be precipitated on the cell surface.

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REPRODUCTION OF VENEZUELAN EQUINE

ENCEPHALOMYELITIS VIRUS AT LOW IONIC STRENGTH

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A study was carried out of the effect of low ionic strength on the reproduction of Venezuelan equine encephalomyelitis [VEE] virus. Data obtained showed that low ionic strength inhibits in reverse the process of virus release from the cell at all stages of virus reproduction. An examination was carried out of the kinetics of the virus escape from cells with a change of ionic strength and the possibility of employing low ionic strength to accumulate intracellular virus in large quantities. It was established that under hypotonic conditions synthesis of viral RNA and proteins in an infected cell occurs at a normal rate. An analysis was carried out of viral structures formed in cells in a normal and hypotonic state. It was shown that with low ionic strength in a logarithmic period viral ribonucleoproteins formed and accumulated in the cells which were probably bound with membranes slower than under normal conditions. Data is presented showing that at the conclusion of the viral cycle in a hypotonic medium intermediate virus forms accumulate on the membranes and

apparently virions with proper sedimentation constants are formed which under these conditions can be precipitated on the cell surface.

The process of releasing arboviruses from cells depends on a number of medium factors among which ionic strength plays an important role (5, 10, 11). The mechanism of the action of low concentrations of salts on reproduction of viruses is still unclear. According to some investigators (6, 10, 12) a change occurs here in the physico-chemical composition of the membranes which in turn leads to a disturbance in the final stages of virus maturation. Proceeding from this it should be expected that in the instances where virus formation occurs on cell membranes the low ionic strength would normally lead to a decrease in virus release. Such a situation has been noted in two studied arboviruses (6, 13) and a microvirus (5).

Previously it was shown by us (4), using electron microscopy, that the formation of nucleoides of VEE virus occurs near vacuoles formed in the cytoplasm of infected cells and the formation of supercapsides as a result of the nucleoide gemmation on the walls of cytoplasmic membranes.

The problem posed for this study included an investigation of the influence of low ionic strength on the reproduction of VEE virus. In the formulation of the experiments described below we proceeded from the thesis that although in a state of low ionic strength we did not observe the release of virus into the medium there nevertheless is formation in the cells of nucleoides with proper sedimentation constants. Thus, it is possible with the use of this methodology to accumulate structures in the cells and to separate the processes of the formation of virions and their

release into the medium. The purpose of the present study lay in the experimental verification of these theses.

Materials and Methods. Basic method for formulating the virological experiments.

Methodology for establishing the radioactivity in tissue culture.

The basic method of cultivating the virus in penicillin flasks has been described previously. The number of virus infections amounted to 10-50 BOE/cell. For purposes of a hypotonic medium to cultivate the virus we employed medium 199 diluted 2-1 with bidistilled water. The medium with a normal ionic strength was prepared using a hypotonic medium and a 5 M solution of NaCl. The infectious virus titre was found by the method of an agar patch cover.

In order to detect radioactivity in the tissue culture we employed a method, developed by us, permitting us to combine the basic method of setting up the virological and biochemical experiments with radioisotope technology. The cells were cultivated at the bottom of the flasks for the radioactivity scintillation count, were infected with the virus and tagged precursors -- H^3 uridine and C^{14} aminoacid to a concentration of 5 mkKi/ml. At various stages of the infection the culture fluid was poured off in order to determine the virus titre infectiousness. The cells were washed 3 times with a chilled physiological solution then fixed with a 5 % solution of trichloracetic acid for 15 minutes (while solubilization of the acid solving tracer was taking place) and then washed twice more for 5 minutes in trichloracetic acid. Then the mono-layer was treated for 5 minutes in a ether-alcohol mixture (1-1) to remove lipids. The flasks

were washed separately in alcohol and ether to remove radioactive contaminants on the flask walls, they were dried and filled with 1 ml of toluene scintillator formulated according to the standard prescription. Samples were counted using the Packard-Tricarb Counter. The count spread in parallel specimens did not exceed 10 %.

Secretion of Viral Structures Form the Cells.

Two hours prior to infection the 2-3 day old culture of chick fibroblasts was treated with actinomycin D in a 2 mkg/ml concentration at 37°, then the cells were infected with the virus, 10-20 BOE/cell. Following contact with the virus for 1 hour at room temperature the monolayer was washed off with the Hanks saline solution and the mats were filled with medium 199 at low and normal ionic strength prepared as previously described and containing 5 mkKi/ml of H^3 uridine. Incubation of infected cultures with the tagged precursor was carried out at 37° for 5 and 12 hours. Then the medium was poured off, the cells were washed clean of the medium using a hypotonic solution, NaCL and removed with trypsin prepared with a hypotonic solution. In cases of viral multiplication in the medium with normal ionic strength the cells were washed and removed with a physiological solution of NaCl. The cells were precipitated at 1500 rpm for 10-15 minutes, washed of trypsin and suspended in 2-3 ml of buffer D (type RSB: 10 mM tris, 1mM KCl and 0.1 mM of $MgCl_2$). After this the cells were swollen in a buffer at 0° for 10-15 minutes and disintegrated in a homogenizer. The nuclei were precipitated for 10 minutes at 1500 rpm and the unprecipitated fluid was subjected to fractionation at 10,000 g for 15-20 minutes. The precipitate and the

unprecipitated fluid were used for an analysis in the gradients. The material was kept in liquid nitrogen at - 180°.

Analysis of Structures in a Linear Saccharose Density Gradient

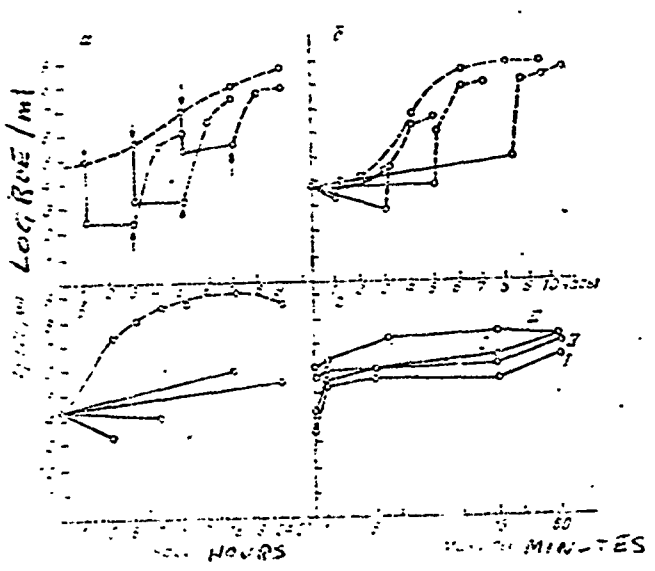
We prepared a linear saccharose density gradient of 15-40 % in buffer D (20 ml). We placed 1-2 ml of the material on the gradient while in some of the experiments the material prior to being placed on the gradient was treated with 10 mM of EDTA (ethylenediaminetetraacetic acid sodium salt). Centrifugation was carried out in a bucket rotor 3x25 ml at 25,000 rpm for 2.5-3 hours in a Superspeed-65 (MSE) ultracentrifuge. The gradient was scooped up from the bottom of the test tube by the method of squeezing 16-32 drops on the LKB sampler with a recording device at 258 nm. In order to calculate the dispersion of radioactivity in the gradient we selected 5 mkl samples from the fractions and placed them on disks of No 4 chromatographic paper (England). After drying the disks were washed 3-4 times in a 5 % solution of trichloracetic acid and then in alcohol and ether. The dry disks were placed in flasks with toluolene scintillator and radioactivity was determined using the Packard-Tricarb Counter.

Results

Action of Ionic Strength on Viral Release from the Cell.

In series 1 of the experiments we studied the release of the infectious virus in a hypotonic medium at various stages of the multiplication cycle. The experiments were formulated under conditions of a single-cycle viral infection which as had been previously pointed out (2) comprised for the virus VEE 6-8 hrs. The experiment was formulated as

follows: the infected cells were initially infected in a medium with normal ionic strength and subsequently at various stages of the infection (1, 3, and 5 hrs) the medium was replaced with a hypotonic. After 2 hours a reverse change of the medium was carried out using the isotonic and we observed the viral production for another 2 hrs. The results of the experiment are presented in Fig. 1a. As can be seen the replacement of the normal medium for the hypotonic led throughout the investigation, following the infection, to an expressed inhibited production of the infectious virus. The phenomenon of submerging the appearance of the virus of low ionic strength was reversible. During the replacement of the hypotonic medium with the isotonic there took place the restoration of the infectious virus titre which soon nearly approached the control values.



[For legend to Figure 1 see p 7]

Figure 1. EFFECT OF LOW IONIC STRENGTH ON VIRUS RELEASE

Dotted line-isotonic medium; Solid line-hypotonic medium; arrows pointed to the nature of the change of the medium; downward-change of the isotonic medium to the hypotonic; up, the reverse; a) substitution of the isotonic medium for the hypotonic was carried out 1, 3 and 5 hours after infection, following this the cells were incubated in a hypotonic medium for 2 hours and then a reverse substitution of the medium took place; b) cells were incubated immediately following infection in a hypotonic medium for 1, 3, 5 and 8 hours and then the ionic strength was brought up to normal at designated periods with the addition of NaCl. Infectious titre of the virus in the medium were identified immediately following the addition of salt and after 1 and 2 hours; c) the infected cells were incubated in a hypotonic medium immediately following infection for 5, 7, 18 and 24 hours; d) the kinetics of the rate of virus release from the cells during the change of the hypotonic medium for the isotonic correspondingly after 5, 7, 18 and 24 hours following infection (lines 1-4 respectively); dots in the drawings represent the infectious titre found after the time shown on the abscissa and following infection (a-c) or after the medium change (g).

Key: 1. Hours, 2. Minutes, 3. Lg BOE/ml.

In subsequent experiments, of this series the cells immediately after infection were incubated in a hypotonic medium and after 1, 3, 5 and 8 hrs the ionic strength was brought up to the normal with the addition of 5 M NaCl. The infected titres of the virus in the medium were calculated immediately following the addition of salt and after 1 and 2 hrs. The results obtained are presented in Figure 1b. As can be seen in Figures 1a and b the presence and the addition of the hypotonic medium during the course of the latent period and in the initial and final phases of the logarithmic period did not disturb the subsequent stages of the formation of the virus. Incubation of the infected cells under hypotonic conditions during the entire reproduction cycle barely reduced the infectious titre of the virus released from the cells with the normalization of the ionic strength in comparison with control. Suppression and restoration of the viral product with changes in ionic strength occurred very rapidly which is very characteristic of processes for releasing the virus from the cells. Five minutes after medium replacement and by the 8th hour of the infection the cells released a quantity of the virus which under normal conditions is synthesized during the course of the entire logarithmic period. Moreover, the greatest restoration effect was observed during a change in the ionic strength in the logarithmic period i. e. when under normal conditions a rapid release of the basic mass of the mature virus from the cells takes place.

The results described coincide with data of other authors (5, 6, 10-12) who had studied the release of the virus under hypotonic conditions and confirm the theses that the action of low ionic strength is linked to the latter stages of the formation of the virus.

In a special series of experiments we studied the kinetics of the rate of release of the virus from the cells following a change of the hypotonic medium to an isotonic after 5, 7, 18 and 24 hrs following infection (Figure 1d). One minute after the replacement of the medium the cells released a significant quantity of the infected virus and this process continued at an accelerated pace for 1 hour after which the rate became normal. The greatest rate of viral release from the cells was observed after 5 and 7 hrs.

In another series of experiments we attempted to explain the duration of the action of suppresion of low ionic strength of the virus release into the medium and to calculate the possibility of using this phenomenon in order to obtain intracellular virus in large quantities. For this purpose the infected cells were incubated in a hypotonic medium for 5, 7, 18 and 24 hrs. Figure 1d shows that only by the 18th hour did we observe an insignificant release of the virus into the medium; this apparently is the result of the death and destruction of a portion of the infected cells. Therefore it is in reality possible with the help of low ionic strength to prevent the release of the virus from the cell for a prolonged period of time and to obtain a large quantity of the intracellular virus.

Synthesis of Viral RNA and Protein under Conditions of Low Ionic Strength

In order to obtain additional data on the mechanism of action of the low ionic strength on the reproductive stages of one virus and to clarify the stages of inhibition we studied the rate and scope of the

inclusion of tagged precursors in viral RNA and protein in a hypotonic and normal medium. During various phases of the infection (1, 3 and 5 hrs) we introduced into the incubation medium, for a 2 hour period, tagged precursors of RNA synthesis and protein. We determined the infectious titre of the virus in the culture medium and radioactivity in the specially treated mono-layer of cells. Figures 2a and b show the curves for the inclusion of tagged precursors in viral macromolecules under conditions of the suppression of the synthesis of cellular RNA by more than 99 %. The synthesis of viral RNA and protein in an infected cell in a hypotonic medium in comparison with the normal was not inhibited, the rate and scope of the inclusion of tagged precursors under hypotonic conditions was even somewhat higher. The nature of the curves in both instances coincided, synthesis of viral RNA increased linearly up to 7 hours while protein synthesis attained its maximum by the third hour and then began to decrease. Under conditions of the absence of the suppresion of the synthesis of cellular RNA (without actinomycin) the inclusion of tagged precursors in a hypotonic medium was somewhat lower than in the isotonic. This probably reflects the suppresion of the cellular synthesis proper in the medium with a lowered salt concentration (Figures 2 c and d). These findings confirm the results of other authors (12) who along with this have shown that the process of releasing the virus from the cells does not require protein synthesis de novo and energy.

These results show that the phenomenon of the suppresion of viral reproduction under hypotonic conditions apparently is not linked with processes of the transcription and translation of viral macromolecules and is observed at much later stages of virus multiplication.

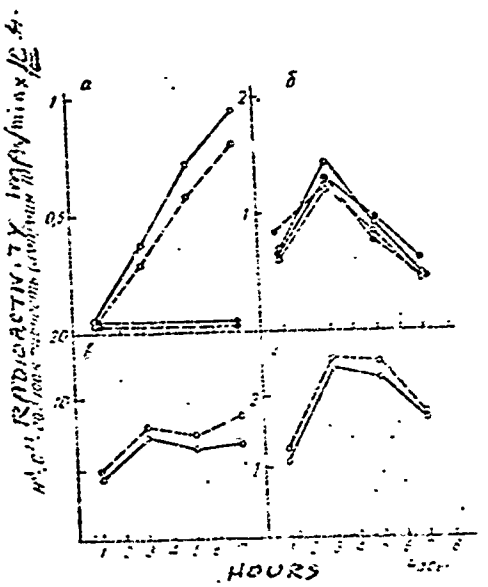


Figure 2. RNA AND PROTEIN SYNTHESIS UNDER CONDITIONS OF LOW IONIC STRENGTH

The dotted line with the clear circles represents the inclusion of tagged precursors in the isotonic medium in infected cells and the dotted line with the black circles represents the inclusion of the tagged precursors in the isotonic medium in non-infected cells. The solid line with the clear circles represents the inclusion of tagged precursors in a hypotonic medium in infected cells and the solid line with the black circles represents the inclusion of tagged precursors in a hypotonic medium in non-infected cells; a) inclusion of H^3 uridine in virus and cell RNA with actinomycin; c) the same without actinomycin D; b) inclusion of C^{14} aminoacids in the protein of infected and non-infected cells with actinomycin D; d) the same without actinomycin D; abscissa represents the length of the analysis (hours following infection).

Key: 1. Hours; 2. H^3-C^{14}-radioactivity (imp/min x 10^{-4}).

Virus Specific Structures Formed in Cells at Low Ionic Strength.

The goal of subsequent research was a study of the effect of low ionic strength on processes of the formation and accumulation of viral structures in the infected cells. The infected cultures were incubated in a hypotonic medium for 5 and 12 hours. Then fractions were obtained from the cells as described in the section on Materials and Methods. These were analyzed in linear saccharose density gradients. Figure 3 shows the profiles of the radioactivity of structures from the fraction obtained by precipitation of the cellular homogenate at 10,000 g. As we had assumed, at low ionic strength an accumulation of structures took place in the infected cells which sedimented in the gradient at the rate of incomplete forms of the virus or nucleoproteids linked with membranes of the cells and contained virus specific RNA. As can be seen from Figure 3, the structures formed by the fifth hour of infection under normal conditions showed in the gradient a homogeneous distribution and under hypotonic conditions sedimented in the form of 2 types of structures 180 S and 210 S. It had been shown previously that constants of viral sedimentation and of viron ribonucleoproteid are equal correspondingly to 260 S and 140 S. Apparently, at low ionic strength there was partial destruction or retardation in the process of viral morphogenesis or interaction of the structures and membranes. By the 12th hour of the infection in a hypotonic medium radioactivity in the zone of the sedimentation of viral structures approximately doubled in comparison with normal conditions while infection increased tenfold (form 5 lg to 6 lg BOE/ml). Moreover, the structures formed in the hypotonic conditions sedimented in the gradient at a greater rate than in the normal medium.

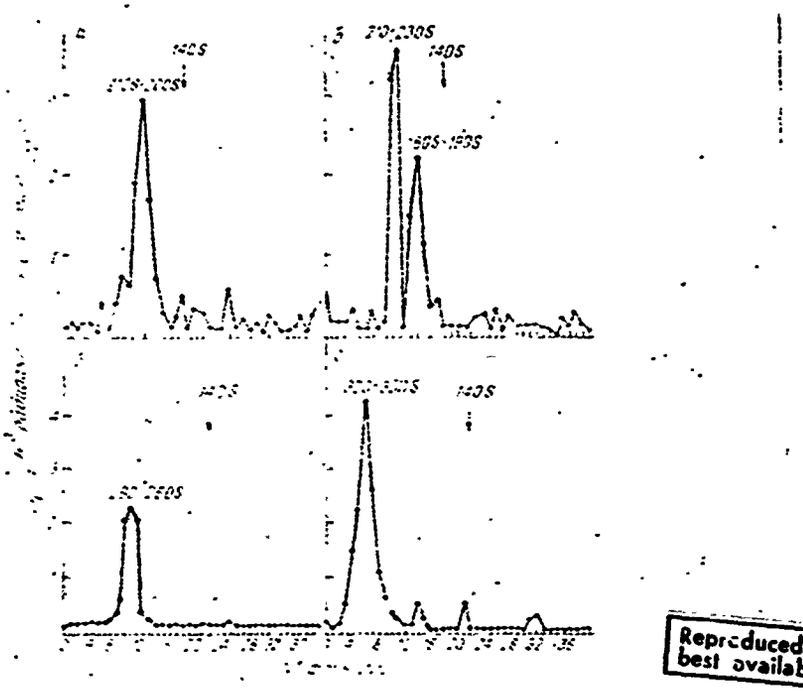


Figure 3. FORMATION OF VIRUS STRUCTURES IN CELLS IN NORMAL AND HYPOTONIC MEDIUM

We analyzed structures obtained by precipitation of cell homogenate at 10,000 g for 15-20 minutes in a saccharose density gradient of 15-40 % (1 ml base of 60 % saccharose) at 25,000 rpm for 2.5-3 hours in a bucket rotor 3 x 20 ml in a Superspeed-65 (MSE) centrifuge. The arrow points to the position of the marker (virion ribonucleoproteid). a) Cultivated in a isotonic medium; b) cultivated in a hypotonic medium 5 hours after infection; c) in a isotonic medium; d) in a hypotonic medium 12 hours after infection.

Key: 1. H^3 radioactivity (imp/min); 2. no of fractions.

As a result of the processing of the VEE structures (Figure 4) with EDTA their sedimentation constants did not decrease. Inasmuch as the virions and ribonucleoproteids are insensitive to this type of treatment there probably took place a dissociation of the structures from the cytoplasmatic membranes. Results of the electron microscope studies of the processes of the formation of the virus in the infected cell (4) showed that in these processes roles are played by the cytoplasmatic vacuoles and membranes which carried the viral nucleoides. A comparison of the position of the structures in the gradient with the sedimentation of the virion ribonucleoproteid secreted from the virus by non-ion detergents and of the purified virus made it possible to assume that by the 5th hour of the infection viral ribonucleproteids separated from the membranes and by the 12 hour more complete forms of the virus also separated. At the same time under hypotonic conditions by the 12th hour of the infection various structures constant in sedimentation were linked with the membranes and apparently represented various stages in the morphogenesis of the virus.

Thus, an analysis of the structures formed in the infected cells under hypotonic conditions showed that the process of interaction of viral nucleoproteids with cell membranes in the logarithmic period of the viral cycle is frequently disturbed or what is more likely retarded. By the 12th hour of infection the cellular membrane accumulates a number of intermediate forms of the virus and there are even formed virions complete in the sedimentation constant; however, their release into the medium under these conditions is not observed.

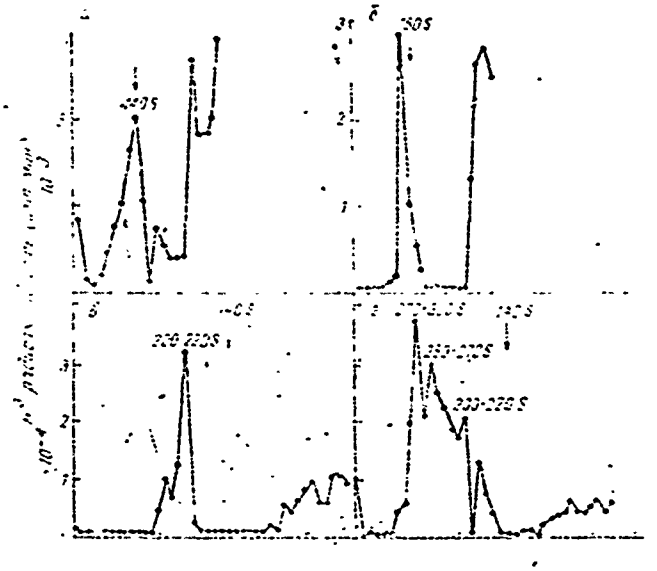


Figure 4. EFFECT OF EDTA ON VIRUS STRUCTURES

Precipitant after centrifugation of the cell homogenate at 10,000 g was treated with 0.01 M EDTA for 5 minutes at 0°. The conditions for the analysis of the structures and the remaining designations are the same as for Figure 3.

It is of interest to note that during an analysis of the structures formed under the hypotonic conditions the fraction of the supernatant following centrifugation at 10,000 g did not show structures with a sedimentation constant of 60-80 S although we observed a significant increase in the radioactivity above the gradient. Similar structures were observed and described in infected cells and according to a number of authors may represent complexes of viral RNA with a small ribosomal subunit (9) or viral specific informosome (1).

Discussion.

As we know, a gradual liberation of hereditary virions into the medium as they mature is normal for arboviruses (8). At the same time the release of mature virions from the cell amounts to a total of 20 sec (7) therefore during titration we normally observe the predominance of the intracellular virus over the intercellular. However, as our investigations showed the process of the release of the virus from the cells is determined to a significant degree by the ionic strength and under the conditions of low ionic strength of the medium we observe a reverse situation with the predominance of the intercellular virus over the intracellular. Therefore only under normal conditions is it possible to judge the dynamics of the formation of the virus in the cells according to the concentration of the culture fluid. Our data show that under hypotonic conditions basic stages of the formation of the virus take place; however, the release into the medium of infectious virions is inhibited.

A comparison of the results obtained by us with data of other investigators (5, 6, 10, 11) permits us to draw certain conclusions regarding the mechanism of the effect of low ionic strength on the reproduction of viruses. Above all the results of the virological studies show that the effect of low ionic strength is manifested at the latter stages of viral reproduction. This conclusion also proceeds from data on the study of the rate of synthesis of viral RNA and proteins of an infected cell under hypotonic conditions. Although the inclusion of tagged precursors into the viral macromolecules cannot as yet be considered as absolute proof of their synthesis according to available data (10, 12), the RNA formed

under these conditions is infectious, sediments in the saccharose density gradient at a normal rate and is included in the composition of viral ribonucleoproteids. The latter, as shown previously in this work, accumulate in the cells at low ionic strength by the 5th hour of infection. Moreover, apparently there is a slowdown in the process of interaction among the nucleoproteids and the cytoplasmatic membranes. Probably on the basis of this process there is the phenomenon of physicochemical nature depending on ionic interaction between proteins of the nucleoproteids and cell membranes. The data obtained by us that by the 12th hour of infection the viral structures in a hypotonic medium linked with the membranes sediment in the saccharose density gradient at a greater rate than structures formed in an isotonic medium may be explained in the first place by an accumulation of structures on the membranes and in the second place by the formation of intermediate forms of the virus which are heavier according to the constant of sedimentation which under these conditions probably precipitate on the surface of the cell and can be rapidly released into the medium at a greater ionic strength. The electronmicroscopic investigation of this phenomenon conducted in earlier studies (6, 13) confirms the data obtained by us as well as the explanation.

Bibliography

1. Belitsina, N. V., et al. Molecular Biology, 1968, no 2, p 727.
2. Zhdanov, V. M., Yershov, F. I., Uryvayev, L. V., Problems of Virology, 1969, no 3, p 292.
3. Ball, C. R., Poynter, R. W. et al. Analyst. Biochem., 1972, v. 46, p 101.
4. Byhovsky, A. F. et al. J. Virol, 1969, v. 4, p 496.

5. Eaton M. D., Scala, A. R., Proc. Soc. exp. Biol. (N. Y.), 1956, v. 92, p 289.

6. Matsumura, T. et al. Ibid., 1972, v. 141, p 599.

7. Mussgay, M., Weibel, J., Virology, 1962, v. 16, p 52.

8. Rubin, H. et al. J. exp. Med., 1955, v. 101, p 205.

9. Sreevalsan, T., Allem, P. T., J. Virol., 1968, v. 2, p 1038.

10. Tolskaya, E. A., Agol, V. I., et al. Virology, 1960, v. 29, p 613.

11. Waite, M. R. F., Pfefferkor, E. R., J. Virol., 1968, v. 2, p 759.

12. Idem. Ibid., 1970, v. 5, p 60.

13. Waite, M. R. F. et al. Ibid., 1972, v. 10, p 537.